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Strategies to Promote Chondrogenesis and Osteogenesis from Human Bone Marrow Cells and Articular Chondrocytes Encapsulated in Polysaccharide Templates

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ABSTRACT

The aim of this study was to synthesize functional *in vitro* and *in vivo* 3-dimensional (3D) constructs using a mix of human mesenchymal populations and articular chondrocytes encapsulated in biomineralized polysaccharide templates. Single-cell–type populations or mixtures of both cell types were encapsulated in alginate/chitosan and cultured within a rotating-bioreactor, perfused bioreactor system, or static conditions for 28 days. Within single cell-type populations, type II collagen immunopositive cells were present within lacunae in rotating-bioreactor capsules, with an increased proportion of metabolically active cells compared with perfused and static constructs. Biochemical analysis indicated significantly increased ($p < 0.05$) DNA and protein in rotating-bioreactor conditions compared with perfused or static. However, in coculture samples, DNA and protein was significantly increased in static cultures owing to the formation of large regions of partially mineralized osteoid. This osteoid was found only in static cultures and when the ratio of human bone marrow cells to chondrocytes was 2:1 or, to a lesser extent, 5:1 ratio capsules. Subcutaneous implantation of capsules into immunocompromised mice also showed optimal osteoid formation when the ratio was 2:1. The current studies demonstrate the pivotal role of robust 3D biomimetic microenvironments and indicate the potential to harness the interactions between different cell types to create specific tissues.

INTRODUCTION

ARTICULAR CARTILAGE DEGENERATION resulting from degenerative diseases or trauma remains a significant healthcare issue. To date, there remains a paucity of clinically viable cartilage formation regimes exacerbated by the fact that cartilage has a limited potential to self-regenerate and an absence of innervation or vascular supply. Similarly, bone loss as a result of trauma, skeletal deficiency, infection, or tumor and subsequent repair or augmentation remains a considerable unmet challenge. Proposed methods to treat osseous and chondral defects are limited and long-term outcomes are unclear, indicating the need for alternative strategies. Examination and exploitation of the interactions between different cell types within a tissue engineering framework to create specific tissues could provide a facile process for new tissue formation.

Stem cells are a population of cells that are able to provide replacement cells for a given differentiated cell type. The use of human mesenchymal stem cells has several advantages as they have unique biological properties, are capable of extensive replication in culture in an undifferentiated state, and can differentiate into multiple mesenchymal lineages, including bone, cartilage, and fat.[1–5] In combination with an

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appropriate cell source, the choice of scaffold is a key component to the success of skeletal tissue engineering. The "scaffold/matrix" must support tissue growth and promote the development of, ideally, mechanically competent and functional tissue.[6] Depending on the desired tissue and the specific application, the scaffold material and its properties will be key. Synthetic materials are appealing as their physical and chemical properties can be modulated and these materials are reproducible. However, it is also clear that some synthetic materials are hydrophobic and if processed under severe conditions may not promote optimal cellular growth and differentiation. Natural materials have gained recent interest as they are structurally similar to the native extracellular matrix of many tissues; exhibit excellent biocompatibility; and cause minimal inflammatory responses, thrombosis, and tissue damage.[7] Two such materials, alginate and chitosan, have been commonly used in a variety of biomedical applications and in assessing the potential of hydrogel scaffolds for cartilage and bone engineering. Chitosan and alginate are natural cationic and anionic polysaccharides, with broad applicability for tissue regeneration as a consequence of their ability to support a variety of cell types,[8] and chitosan has proved an effective scaffold material in cartilage and bone engineering.[9] Lahiji and co-workers have reported chitosan can support the survival of osteoblasts and chondrocytes and the expression of their extracellular components,[10] while Seol and colleagues (2004) reported chitosan promoted growth and mineralized matrix deposition by osteoblasts in culture.[11] Chitosan is structurally similar to glycosaminoglycans (GAGs), molecules that are important in modulating chondrocyte morphology, differentiation, and function, which are found in native articular cartilage.[12] Similarly, alginate hydrogels have been shown to support the survival of osteoblasts and chondrocytes and expression of relevant extracellular components and are robust templates for cell encapsulation, drug delivery, and gene delivery.[8,13] *In vitro* experiments have shown that alginate can stimulate the chondrogenic phenotype of transplanted cells,[14] and, significantly, alginate can promote the synthesis of cartilage-specific macromolecules such as proteoglycans and collagens.[15] Alginate also permits the cotransplantation of multiple cell types, such as osteoblasts and chondrocytes, and appropriate growth stimuli to promote both the osteogenic and chondrogenic phenotype.[16] The ability to localize multiple cell types enabling cell contact and cell factor production/interaction may offer an alternative strategy to manipulate and modulate cell populations.

To facilitate the growth of functional tissue engineered constructs, bioreactor technologies have been employed in a number of previous studies. Bioreactors such as perfused and rotating-wall reactor systems provide efficient and uniform mixing, facilitate the maintenance and diffusion of nutrient levels and pH, and may provide mechanical stimulation and thus improve the formation of functional bone and cartilage tissue. Perfused culture allows fluid flow, high nutrient delivery rates, and the development of true 3-dimensional (3D) cell growth and organization. Perfused culture has also been shown to enhance cartilage tissue engineering as demonstrated by good matrix formation and the promotion of mature chondrocyte phenotype.[17] The rotating-wall bioreactor has efficient mass transfer properties, and the permissive environment encourages chondrogenesis.[18] Other advantages include homogeneous cell distribution and minimal damaging turbulence and shear forces.[19]

The aim of this study was to promote osteogenic and chondrogenic differentiation and to synthesize functional *in vitro* and *in vivo* 3D constructs using a mix of human mesenchymal progenitor and articular chondrocyte populations encapsulated in unique biomineralization polysaccharide templates. Harnessing the potential of interactions between different cell types to create specific tissues and exploitation of the mechanisms involved in transition between cartilage and bone phenotype may offer significant potential in the generation of functional 3D tissues for musculoskeletal application.

MATERIALS AND METHODS

All tissue culture and biochemical reagents were obtained from Sigma-Aldrich U.K. unless indicated otherwise. Human recombinant transforming growth factor-β3 (TGF-β3, cat. no. PF073) was purchased from Calbiochem (Merck Biosciences, U.K.), as was the anti-type II collagen antibody (rabbit polyclonal, cat no. 234187). The anti-type I collagen (rabbit polyclonal, LF67) was a gift from Dr. Larry Fisher (NIH, Bethesda, MD). The rotating-wall bioreactor was purchased from Cellon (Arlon, Luxembourg). Tissue culture reagents were purchased from Gibco/BRL (Paisley, Scotland, U.K.).

Cell culture

Human bone marrow and human femoral head samples were obtained from hematologically normal patients undergoing routine total hip replacement surgery. Samples were used with approval from the Southampton Hospital Ethics Committee (LREC194/99). Human bone marrow cells were isolated as previously described.[20] Bone marrow cells were maintained in 10% α-minimum essential medium (α-MEM) in a humidified incubator at 37°C and 5% carbon dioxide (CO_2).

The isolation of human articular chondrocytes was performed by slicing off 2- to 4-mm^3 pieces of cartilage that were digested overnight in collagenase B (10 mg/mL, Roche Diagnostics, Lewes, U.K.). This solution was then passed through a 70-μm filter and centrifuged at 1400 rpm for 5 min. The resulting cell pellet was suspended in an appropriate volume of α-MEM supplemented with 10% fetal calf serum (FCS) and ascorbate-2-phosphate (100 μm). Cells were cultured as a monolayer in humidified atmosphere at 37°C and 5% CO_2 until confluent and harvested for alginate encapsulation.

Preparation of alginate/chitosan microcapsules with encapsulated cells

Ultrapure alginate (NovaMatrix, Drammen, Norway) (0.2 g) was added to 0.09 g of sodium chloride and 0.3g d-sodium hydrogen orthophosphate (211 mM) and dissolved in 10 mL of distilled water. Before the addition of cells, 10 ng/mL of TGF-β3 was added to the alginate solution. Chitosan (3 g) was added to 1 g of calcium chloride (50 mM), 3 mL of acetic acid and 200 mL of distilled water. Appropriate cells were trypsinized and centrifuged and the alginate solution added to the cell pellet and vortex-mixed to ensure thorough mixing and even distribution of cells throughout the alginate. Droplets (100 μL containing approximately 4×10^5 cells) were dispensed onto the surface of the chitosan solution in a Petri dish. Capsules, approximately 5 mm in diameter, were left in the chitosan in a covered Petri dish for 1 h, following self-assembly, for the attachment of the chitosan shell to occur,[21] and were subsequently washed 3 times in α-MEM media. Capsules were held in media supplemented with 10 nM dexamethasone, 100 μM ascorbate-2-phosphate, and 1×ITS premix (insulin—10 μg/mL, transferrin—5.5 μg/mL, selenium—5 ng/mL) for 24 h and subsequently placed either in 6-well plates, in rotating-bioreactor vessels, or into the flow chambers of the perfused bioreactor. Capsules were encapsulated with human bone marrow cells, human articular chondrocytes, or a mixture of the 2 cell types.

Bioreactor technology

Synthecon rotating-wall bioreactor. The Synthecon rotating-wall bioreactor (USA Synthecon, Houston, TX) consists of a 50-mL disposable rotating vessel that allows the constant movement of samples through media (Fig. 1A). Alginate samples ($n=6$) were placed into the disposable vessel via an access port and then 50 mL of media was added to fill the vessel, making sure not to allow the presence of any air bubbles, as this can affect the orbit of the scaffold within the vessel. Media changes were performed every 7 days via the syringe ports, with the addition of 20 mL of fresh media into one, and the removal of waste from the second.

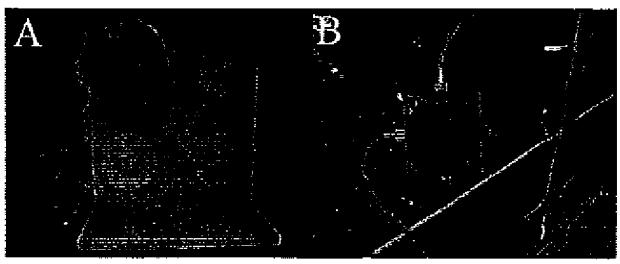


FIG. 1. Rotating-wall bioreactor vessel (**A**) and perfused bioreactor system showing flow chamber connected to silicon tubing to allow continual flow of fresh nutrients through the system (**B**). Color images available online at www.liebertpub.com/ten.

Perfused bioreactor. The perfused bioreactor consists of a central flow chamber in which the scaffold and cells are placed (Fig. 1B). Each reactor has 4 inlet/outlet ports, 2 above the chamber and 2 below that are attached with silicon tubing to a bottle of fresh media and a waste container to allow the continual delivery of fresh media to the samples and constant removal of waste media. The silicon tubing allows gas exchange and prevents any microorganisms gaining access into the central chamber. A valve controls the flow rate, and samples were cultured in this system at a flow rate of 1 mL/h, as this was found to be optimal for the alginate and a faster flow rate damaged the capsules. Samples were also cultured in static culture in 6-well plates with media changes every 2–3 days.

In vivo *studies*

Polysaccharide capsules with and without human bone marrow cells or in combination with articular chondrocytes were prepared (4 per group) and implanted subcutaneously into athymic *MF1-nu/nu* mice (20–24 g, 4–5 weeks old, Harlan, U.K.). After 28 days, the polysaccharide capsules were harvested, and fixed in 50% alcohol formaldehyde with 1% calcium chloride overnight. Samples were processed, sectioned, and stained for Alcian blue/Sirius red, von Kossa, and type I collagen as previously described.[22]

Live/dead staining

The viability of cells was examined using the fluorescent dyes Cell Tracker Green™ (CTG) which identifies metabolically active cells (green) and ethidium homodimer-1 (Invitrogen, Paisley, U.K.) which labels necrotic cells or cells with damaged membranes (red), as previously described.[22] The fluorescence was visualized via confocal microscopy.

Histological analysis

Alginate capsules were fixed overnight in 50% alcohol formaldehyde with 1% calcium chloride, dehydrated in ethanol, cleared in chloroform, and embedded in paraffin wax. Sequential sections were cut at 7 μm, dewaxed in Citroclear, rehydrated, and stained with one of the following techniques:

Alcian blue/Sirius red. After nuclear staining with Weigert's hematoxylin (10 min), proteoglycans were stained with Alcian blue 8GX (0.5% in 1% acetic acid; Merck, Poole) for 5 min, followed by 1% molybdophosphoric acid (20 min) and Sirius red F3B (0.1% in 30% picric acid; Merck, Poole) for 1 h to detect collagen. Slides were mounted in DPX.

Von Kossa. Sections were treated with 1% $AgNO_3$ under UV light (20 min), fixed with 2.5% sodium thiosulfate

(8 min), counterstained with Alcian blue and van Giesen, and mounted in glycerol jelly.

Immunohistochemistry

After blocking endogeneous peroxidase activity with 3% hydrogen peroxide (5 min), the sections were incubated with the primary antibody at room temperature for 1–3 h, followed by 1 h with biotinylated anti-rabbit IgG (Dako, 1:200), then 30 min with ExtrAvidin conjugated peroxidase (Sigma, 1:50). The reaction product was visualized with 3-amino-9-ethylcarbazole (AEC) in acetate buffer containing 0.015% hydrogen peroxidase. Sections were mounted in glycerol jelly. Control slides were not treated with primary antibody, but were otherwise the same as the other samples.

Biochemical analysis

Alginate capsules were dispersed in 300 μL of 0.05% Triton, and cells were lysed by freeze–thawing 3 times. DNA was quantified via the picoGreen dsDNA Quantitation Assay (Invitrogen, Paisley, U.K.). This assay is an ultra-sensitive fluorescent nucleic acid stain of double-stranded DNA. Plates were read on an FLx cytofluor microplate reader. Total protein was measured using the Bio-Rad protein assay and samples were read at 750 nm on a ELx800 spectrophotometer. Osteocalcin was measured using the Intact Human Osteocalcin Eliza Kit (Biomedical Technologies, Stoughton, MA). The Sircol Collagen Assay Kit (Biodye Science, Biocolor, Newtownabbey, N. Ireland) was used to analyze collagen levels. Samples were read at 540 nm.

Statistics

Statistical significance was analyzed using the unpaired *t*-test and ANOVA, and all results are shown as means ± SD. Samples were run in triplicate for the biochemical assays. Statistical analysis was performed using GraphPad Instant Software (GraphPad Software, San Diego, CA). All experiments were repeated at least twice.

RESULTS

Chondrogenic differentiation of single-cell types cultured within rotating bioreactors

After 28 days of culture in the rotating-wall bioreactor, cells were distributed evenly throughout the alginate capsules and had developed lacunae. This was observed in samples encapsulated with human bone marrow cells (Fig. 2A) as well as chondrocytes (Fig. 3A). Capsules from perfused and static conditions were less organized and cells were not evenly distributed throughout the capsule, but were located primarily around the periphery (Figs. 2 and 3B and 2 and 3C). A high proportion of metabolically active cells, as evidenced by Cell

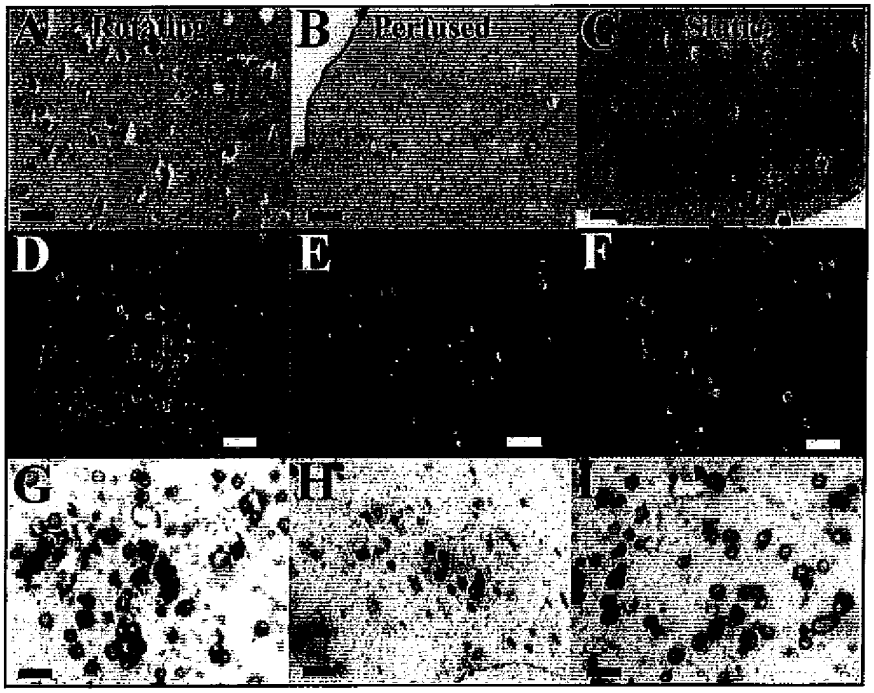


FIG. 2. Alginate polysaccharide capsules encapsulated with human bone marrow cells at day 28. Alcian blue and Sirius red staining of rotating-wall bioreactor (**A**), perfused bioreactor (**B**), and static culture sections (**C**). Cell viability in samples from the rotating-wall bioreactor (**D**), perfused bioreactor (**E**), and static conditions (**F**). Presence of type II collagen in samples from the rotating-wall (**G**), perfused (**H**), and static culture conditions (**I**). Scale bar = 50 μm. Color images available online at www.liebertpub.com/ten.

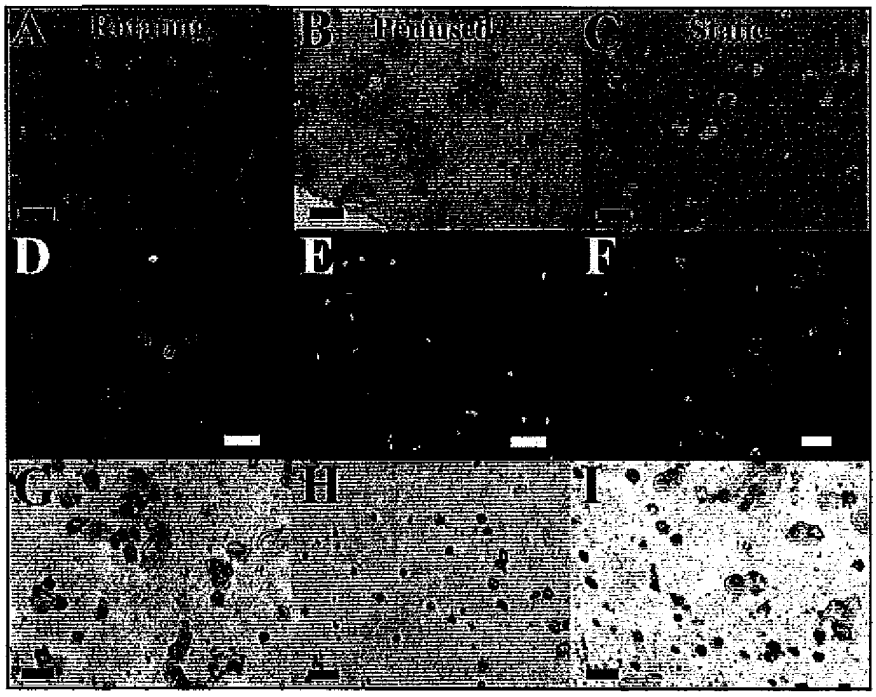


FIG. 3. Alginate capsules encapsulated with human articular chondrocytes after 28 days in culture. Alcian blue and Sirius red staining of samples from the rotating-wall bioreactor (**A**), perfused bioreactor (**B**), and static conditions (**C**). Live/dead staining of rotating-wall bioreactor samples (**D**), perfused samples (**E**), and static samples (**F**). Type II collagen staining in samples from the rotating-wall (**G**), perfused (**H**), and static culture conditions (**I**). Scale bar = 50μm. Color images available online at www.liebertpub.com/ten.

Tracker Green™ reactivity, were present within samples from the rotating-wall bioreactor (Figs. 2 and 3D), whereas capsules from the perfused and static conditions contained more necrotic cells, as demonstrated by an increase in cells that stained red with ethidium homodimer-1 (Figs. 2 and 3E, F); in addition, the cells were of a much smaller size within perfused bioreactor constructs. This was consistently demonstrated in both the human bone marrow cultures and chondrocyte cultures, indicating greater cell viability when cultured within the rotating-wall bioreactor compared to perfused and static conditions. All marrow-stromal derived cells and articular chondrocytes were immunopositive for type II collagen, the cartilage-specific collagen, in cultures from each condition after the 28-day culture period (Figs. 2 and 3G–I), although there was a slightly higher proportion of cells in the rotating-wall cultures. However, positive type II collagen staining was observed only within the cells and not in the matrix, suggesting an early stage of chondrogenesis.

Osteogenic differentiation in mixed-cell cultures in static conditions

When human bone marrow cells and chondrocytes (2:1 ratio) were cocultured for 28 days, numerous cells were present within distinct lacunae throughout the rotating-wall capsules (Fig. 4A), whereas in the perfused bioreactor samples fewer cells were present, located primarily towards the periphery of the capsules, and a lesser proportion had developed lacunae (Fig. 4B). Just as in single cell-type samples, more metabolically active (CTG-positive) cells were found within both the central and peripheral regions in rotating-wall samples (Fig. 4D) in comparison with perfused samples (Fig. 4E). However, in the coculture samples from static conditions, large regions of a Sirius-red–positive matrix encapsulating numerous cells was observed (Fig. 4C). This was not detected in any coculture samples from the perfused or rotating-wall bioreactor systems but within all coculture static constructs. Cells within the structure were highly metabolically active, as confirmed by live/dead staining (Fig. 4F). Immunocytochemistry demonstrated the presence of type I collagen in the cells and matrix (Fig. 4G) and part of the matrix was mineralized, as shown via von Kossa staining (stained brown-black in Fig. 4H). Birefringence further confirmed the presence of organized collagen within the alginate matrix (Fig. 4I), indicating the matrix had the characteristics of osteoid with some mineralized bone matrix.

Biochemical analysis of single-cell–type and cocultures

To determine cell proliferation and cell activity, single-cell–type and mixed cell population samples were analyzed for DNA and total protein content. For the perfused and

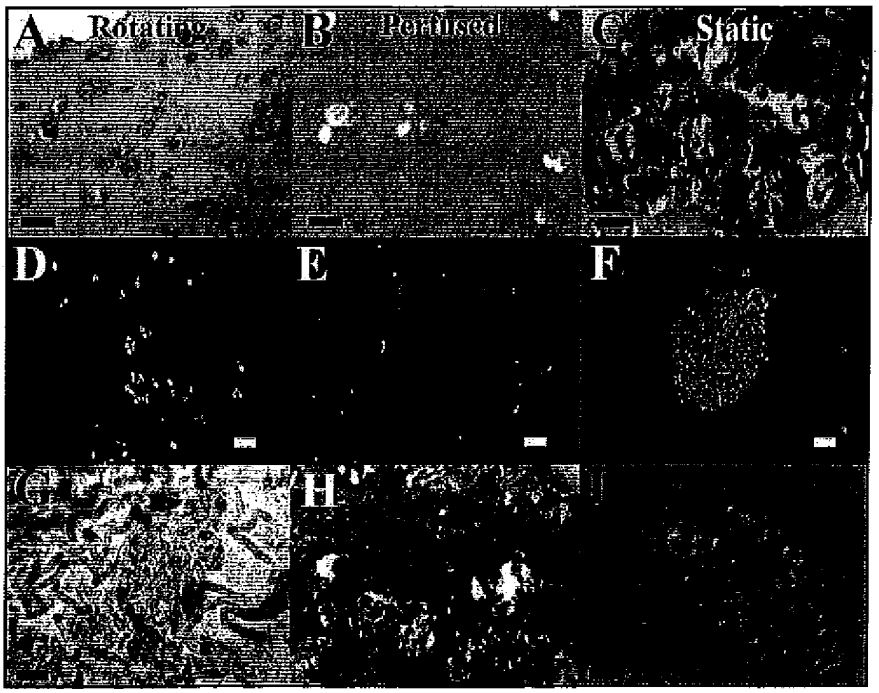


FIG. 4. Bone marrow cells were mixed with chondrocytes in a ratio of 2:1 and cultured for 28 days. Alcian blue and Sirius red stained sections from the rotating-wall bioreactor indicating development of cells within lacunae (**A**), perfused bioreactor (**B**), and static conditions demonstrating the development of large regions of mixed proteoglycan and collagen staining (**C**). Cell viability in samples from the rotating-wall (**D**), perfused (**E**), and static conditions (**F**). Immunohistochemistry indicated extensive regions of type I collagen staining in samples from static conditions (**G**), von Kossa staining indicated matrix mineralization and osteoid formation (**H**), and polarized light microscopy demonstrated organized collagen (**I**). Scale bar = 50 μm.

static cultures, there was no difference in DNA content after 14 or 28 days in comparison with day 0 (Fig. 5A). However, after 28 days the DNA content had significantly increased ($p < 0.05$) in human bone marrow cultures from the rotating-wall bioreactor. Total protein content decreased in perfused and static cultures after 14 and 28 days compared with preculture (Fig. 5B). However, this was not the case for the rotating-wall bioreactor, where protein synthesis had significantly increased ($p < 0.05$) after 14 and 28 days (Fig. 5B), consistent with the increased DNA content. By contrast, in chondrocyte cultures, perfused conditions resulted in significantly higher DNA ($p < 0.05$) than in rotating-wall and static and there was actually a decrease in cell proliferation in the rotating-wall bioreactor capsules after 14 and 28 days, suggesting an increased incidence of cell death (Fig. 5C). Overall total protein content decreased after 14 and 28 days; however, this decrease was less marked in the rotating-wall constructs (Fig. 5D), suggesting that, although there was a decrease in cell proliferation in rotating samples, those cells that were proliferating demonstrated higher protein synthesis in comparison with perfused and static. For coculture samples, after the 28-day culture period, although there was a decrease in DNA in all conditions, there was significantly increased ($p < 0.05$) DNA in static cultures compared with perfused and rotating-wall bioreactors (Fig. 5E). Protein was significantly increased ($p < 0.05$) in all conditions after 28 days in comparison with day 0. In addition, there was a significant increase ($p < 0.05$) in static cultures in comparison with the perfused and rotating-wall bioreactor systems (Fig. 5F). This may be due to the large amounts of osteoid formed in these cultures.

Development of osteoid depends on the ratio of bone marrow cells to chondrocytes

To determine whether the ratios of the 2 cell types influenced the development of osteoid tissue, the ratios of bone marrow cells to chondrocytes were varied from 1:10 to 10:1. At ratios of 1:10, 1:5, and 1:2, cells were individually present within lacunae with no aggregation (Fig. 6A). At a ratio of 1:1, regions of clustered cells of cells were present throughout the capsules, but without elaboration of an extracellular matrix (not shown). Within all 2:1 ratio samples, large regions of mixed staining, for both fibrous collagen and proteoglycans, were found (Fig. 6B). Part of the matrix was histologically similar to osteoid as evidenced morphologically and by the extensive Sirius red staining. Within the 5:1 ratio capsules, smaller regions of collagenous matrix had also formed (Fig. 6C), but to a

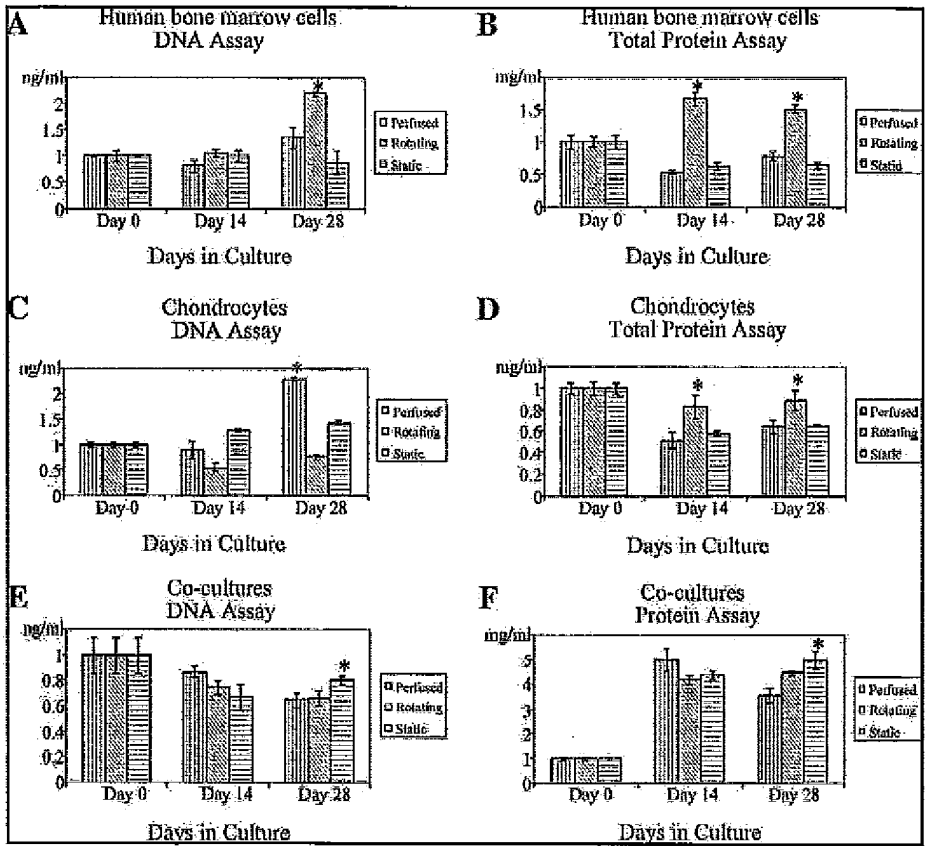


FIG. 5. Biochemical analysis of polysaccharide capsules at days 0, 14, and 28. Human bone marrow cultures demonstrated significantly increased ($p < 0.05$) DNA and protein after 28 days in samples from the rotating-wall bioreactor than perfused or static (**A, B**). Chondrocyte cultures indicated a significant increase ($p < 0.05$) in DNA in perfused samples after 28 days (**C**) and a significant increase ($p < 0.05$) in protein in rotating samples after 14 and 28 days in comparison with perfused and static (**D**). Mixed cell population samples demonstrated significantly increased ($p < 0.05$) DNA and protein in samples from static conditions after the 28-day culture period (**E, F**). Results are shown as an increase/decrease from day 0. All samples were run in triplicate and results are expressed as means ± SD.

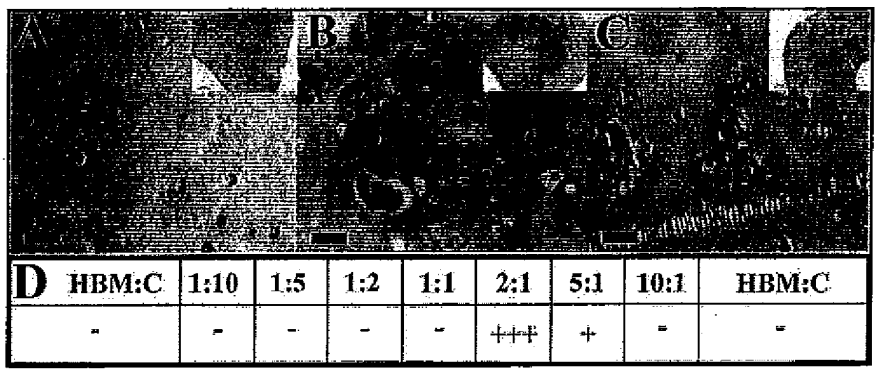
lesser extent than with the 2:1 ratio. In addition, a number of regions of clustered cells without matrix were present, just as for the 1:1 ratio. Osteoid formation or large cell aggregates were not observed in any mixed-cell population capsules other than 2:1 and 5:1 ratios, as indicated in the table within Fig. 6D.

Biochemical analysis of mixed population samples

To determine whether biochemical data supported the histological findings, total DNA, protein, and collagen content as well as osteocalcin levels were measured after a 28-day culture ($n = 6$ for each experimental group). DNA was significantly increased ($p < 0.05$) in mixed-population samples at ratios of 2:1 and 5:1 in comparison with all other groups (Fig. 7A). Protein synthesis was significantly ($p < 0.05$) increased in the 2:1 capsules in comparison with all other cell combinations (Fig. 7B). Significantly increased ($p < 0.05$) collagen production was demonstrated in the 2:1 and 5:1 ratios compared with all other groups. As the proportion of human bone marrow cells increased within the capsules, the total collagen content also increased and peaked at the 2:1 ratio, with a decrease at the 10:1 ratio (Fig. 7C). This pattern was also observed for osteocalcin, a late marker of the osteogenic phenotype (Fig. 7D).

Bone formation in alginate capsules implanted subcutaneously

To determine whether the *in vitro* results could be replicated *in vivo*, alginate capsules containing human bone marrow cells only, chondrocytes only, or a 2:1 mixture of both were implanted subcutaneously into MFI immunodeficient nude mice. The alginate capsules remained intact after the 28-day implantation period and were surrounded by extensive vascularization (Fig. 8A). Metabolically active cells were present throughout all capsules, including the center and the periphery of the samples (Fig. 8B). As for the *in vitro* cultures, cells were present within lacunae



D HBM:C	1:10	1:5	1:2	1:1	2:1	5:1	10:1	HBM:C
-	-	-	-	-	+++	+	-	-

FIG. 6. Alcian blue and Sirius red staining of histological sections of mixed-population capsules revealed the development of chondrocytic cells within lacunae in all cell combinations (**A**). Extensive regions of osteoid formation were demonstrated in the 2:1 ratio capsules (**B**) and small areas of osteoid were demonstrated in the 5:1 ratio capsules (**C**). All samples were identified as having either no osteoid development (–), small areas of osteoid (+), or large regions of osteoid formation (+++) (**D**). Scale bar 50 = μm. Color images available online at www.liebertpub.com/ten.

(Fig. 8C). However, in contrast with *in vitro* culture, capsules containing bone marrow cells only also developed large regions of osteoid-like tissue, which stained with Sirius red (Fig. 8D), was positive for type I collagen (Fig. 8E), and contained mineralized matrix (Fig. 8F), which was birefringent when viewed with polarized light (Fig. 8G). Such osteoid-like tissue was not observed in capsules containing human articular chondrocytes, in which cells were present within lacunae, but had not formed any matrix (Fig. 8H), similar to the *in vitro* cultures. Within the 2:1 coculture constructs, large regions of osteoid formation were again observed, as confirmed by Alcian blue and Sirius red staining (Fig. 8I), immunocytochemistry for type I collagen in both encapsulated cells and surrounding matrix (Fig. 8J), von Kossa staining for mineralization (Fig. 8K), and collagen birefringence (Fig. 8L). No cartilage or bone formation was observed in *in vivo* "alginate only" samples (data not shown).

DISCUSSION

The limited potential for cartilage to self-regenerate and the absence of clinically viable cartilage formation regimes highlights the need for alternative strategies for the formation of cartilage. Thus the initial aims of this study had been to

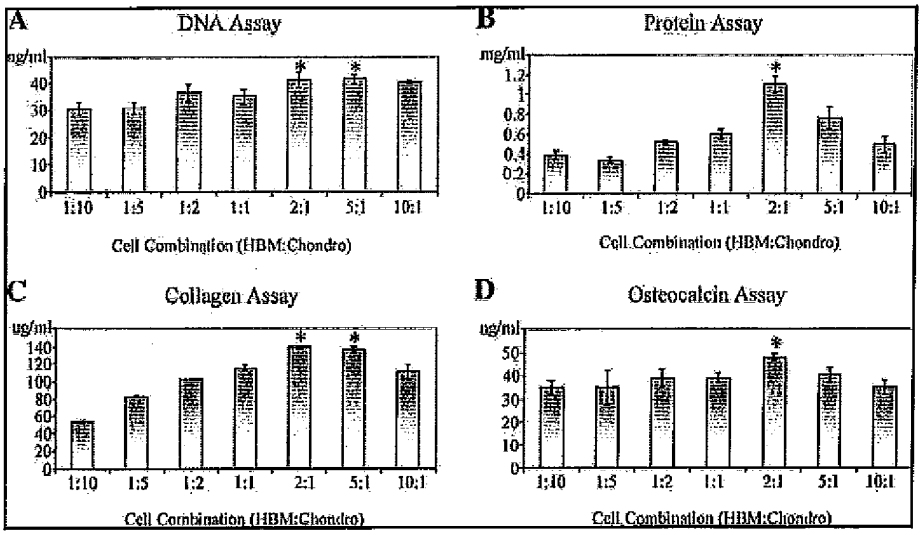


FIG. 7. Biochemical analysis of mixed-population samples after 28 days. Significantly increased ($p < 0.05$) DNA was observed in the 2:1 and 5:1 ratios in comparison to all other cell combinations (**A**). There was a significant increase ($p < 0.05$) in protein in the 2:1 ratio samples (**B**). Collagen was significantly increased ($p < 0.05$) in 2:1 and 5:1 ratios (**C**). There was also an increase in osteocalcin in the 5:1 ratio samples in comparison with all other mixed-population samples (**D**). All samples were run twice in triplicate and results are expressed as means ± SD.

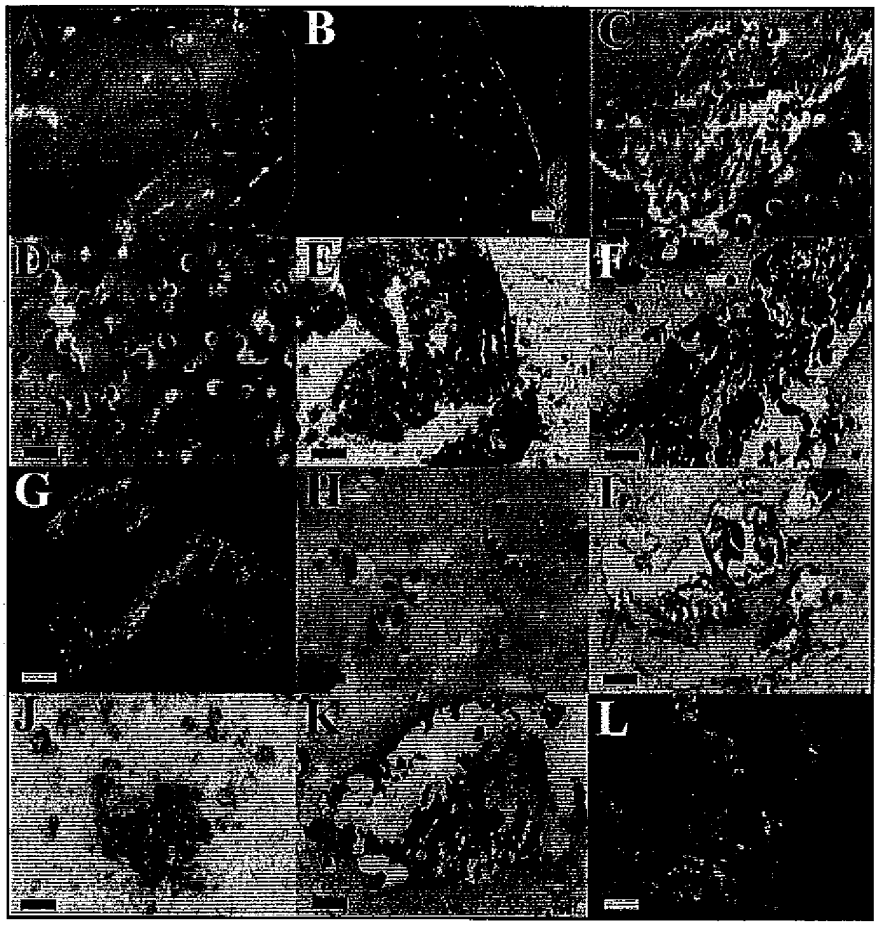


FIG. 8. *In vivo* capsules after the 28-day implantation period. Alginate capsules surrounded by blood vessels (**A**). Cells within capsules were viable after the 28-day implantation (**B**). Alcian blue and Sirius red staining of human bone marrow samples revealed cells within lacunae (**C**) and bone formation (**D**). Immunological staining demonstrated large regions of type I collagen (**E**). Extensive regions of matrix mineralization were confirmed by von Kossa staining (**F**) and organized collagen indicated by polarized light microscopy (**G**). Chondrocyte encapsulated samples indicated the development of chondrocytic cells within lacunae (**H**). Coculture samples revealed large regions of mixed staining, positive for proteoglycan and collagen (**I**). Immunological staining indicated there were regions of matrix and individual cells positive for type I collagen within coculture samples (**J**). Von Kossa staining revealed a region of mineralized matrix (**K**) and polarized microscopy indicated there was an area of organized collagen within the coculture capsules (**L**).

optimize conditions for engineering cartilage tissue from either human articular chondrocytes or human bone marrow cells (or combinations of both), embedded within a mineralized alginate/chitosan capsule and maintained either in static cultures or in rotating-wall or perfused bioreactors. The novelty of the capsules used relates to mineralization of the chitosan outer layer, which serves to enhance structural support, providing a robust structure resistant to rupture by the forces generated within a bioreactor. In previous studies we showed the ability to modulate the structural composition of the polysaccharide capsules to create highly mineralized capsules (through use of increased calcium and phosphate) versus lightly mineralized capsules (lower calcium and phosphate ratios in the alginate and chitosan solutions), providing robust templates resistant to mechanical disruption within bioreactors over at least 28 days.[8] Initial unpublished studies have shown capsules with a chitosan (calcium phosphate) shell remain intact after loading with 0.15 N compressive load whereas capsules without a shell collapse. Further, Leveque and colleagues[21] showed that mechanical resistance to fracture using a simple shear test was significantly increased at a defined amount of calcium phosphate present in the shell compared to un-mineralized capsules. Ongoing work is aimed at more rigorously assessing the mechanical properties of these different constructs using dynamic compression and shear testing. In addition, it is highly likely calcium and phosphate present in the biomineralized capsules serve to promote cell biomimeralization and matrix mineralization processes, as in previous studies *in vivo* capsules with insignificant levels of calcium phosphate mineralization readily rupture and disintegrate before encapsulated human cell populations can synthesize and

organize into tissue (D.W.G. and R.O.C.O., unpublished data).

It had previously been reported that the encapsulation of cells within alginate provided a useful model for studying chondrogenesis, as alginate not only favored differentiation but also maintained the chondrogenic phenotype in both *in vitro* and *in vivo* culture conditions.[16,23] In addition, alginate appeared to facilitate the synthesis of cartilage-specific molecules such as proteoglycans and type II collagen and provided mechanical integrity to the cells and developing tissue.[15,24] The present studies confirmed that alginate is a suitable environment to maintain cell viability. Further, we have demonstrated that, for the culture of human bone marrow cells, under the conditions examined, the rotating-wall bioreactor system was preferable to the perfused bioreactor system and to static culture conditions, as indicated by increased cell viability, increased numbers of cells, and significantly higher DNA and protein synthesis. The cells had formed lacunae within the alginate matrix and were immunopositive for type II collagen, which suggested that the cells had differentiated along the chondrocytic lineage, but had not yet produced a cartilage matrix. Previous bioreactor studies also found that rotating-wall bioreactor conditions resulted in higher growth rates, increased viability, and larger "tissue-like" constructs, as this environment encouraged the aggregation of chondrocytes to form tissue.[19,25] This success has been attributed to the fact that it is a low-shear, high-diffusion bioreactor with constructs falling through the medium while the flow acts in the upward direction, keeping them suspended but also exerting a slight shear force.[26] Perfused bioreactor systems have been developed to eliminate the possibility that mechanical mixing may induce unwanted shear gradients and to improve mass transfer.[25] Although perfused culture has been shown to increase bone growth by providing optimum nutrient and gas supply to the cells, there is limited information regarding the effects of this bioreactor type on chondrogenic cultures. Chondrogenesis is less dependent on high oxygen tensions than osteogenesis, as native chondrocytes survive under hypoxic conditions, and cartilage generally has a low cell density.[27] Therefore, under the conditions examined, the perfused bioreactor system will require further optimization for cartilage formation.

The key observation from this study is the interaction of human bone marrow stromal cells and articular chondrocytes following coculture. Encapsulation with both human bone marrow cells and human articular chondrocytes at a ratio of 2:1 respectively, cultured under static conditions, resulted in large regions of fibrous collagen, positive for type I collagen and histologically similar to osteoid. This was demonstrated in all coculture 2:1 samples in static conditions, but not in any bioreactor samples, suggesting that although the mechanical forces that are experienced in bioreactor culture may be conducive to cartilage formation, it is actually static culture that appears to support osteogenesis in the alginate polysaccharide capsules. The demonstration of *in vivo* analysis, biochemical analysis of enhanced protein synthesis, DNA synthesis, as well as collagen synthesis in the 2:1 and 5:1 constructs in comparison with all other cell ratios suggests a higher proliferation rate for the cells within the 2:1 capsules. However, the interaction between the 2 cell types is clearly essential as no single-cell type construct demonstrated osteoid formation *in vitro*. This suggests the presence of articular chondrocytes provides a necessary factor/cell contact in this process. It is also possible the chondrocytic population promotes the proliferation and differentiation of the human bone marrow cells toward the osteogenic phenotype, even when subjected to chondrogenic conditions. Alsberg *et al.* (2002) encapsulated a combination of bone marrow cells and chondrocytes in a ratio of 2:1 respectively in alginate. Results from their studies indicated regions that closely resembled cartilage and an increase in cell number, density and mineral content over time. In addition, in their cotransplant group constructs, there were areas that contained structures histologically resembling growth plates and areas of mature bone formation. The results outlined in this study were not repeated in the current study although it is important to note that (1) cells used in the Alsberg study were of a rat and calf origin and (2) samples were examined after 26 weeks *in vivo*. The current studies involved the use of human mesenchymal progenitors and were analyzed after 4 weeks. Nevertheless the current studies and data from Alsberg and co-workers support the importance of elucidating the role of cell–cell interactions and delineation of the specific cell interactions resulting in specific tissue (i.e., osteoid) formation.

In summary, the current studies outline the potential of alginate polysaccharide capsules in combination with rotating bioreactors in the generation of cartilaginous constructs. We have demonstrated that rotating-wall bioreactors are preferable to static and perfused culture under the conditions examined and the importance of coculture utilizing individual and mixed human mesenchymal progenitor populations coupled with innovative polysaccharide templates to promote osteogenic and chondrogenic differentiation in *in vitro* and *in vivo* samples. These studies demonstrate the pivotal role of 3D biomimetic microenvironments and the modulation and potential to harness the interactions between different cell types to create specific tissues. Exploitation of the mechanisms involved in transition between cartilage and bone phenotype will be important to understand further the mechanisms involved in directed tissue differentiation and formation with important therapeutic implications therein for orthopaedic tissue repair.

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